METABOLISM OF TNT ASSOCIATED WITH ROOTS OF HIGHER PLANTS

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ABSTRACT Contamination with 2,4,6-trinitrotoluene (TNT) is widespread at sites of past munitions manufacture. It is moderately toxic to plants and highly toxic to some animals. Some aquatic plant species have been observed to promote degradation of TNT. The degradative activity is associated with the root systems but products are found in the surrounding medium. We have examined a range of sixteen genera of non-aquatic plants grown hydroponically to determine whether the promotion of TNT degradation is a general phenomenon of plant root systems. All tested species showed the ability to reduce TNT extensively. Rates varied several fold when calculated on a wet weight of tissue basis. Isolated roots, disrupted roots, and root extracts generally showed less activity than intact roots attached to the plants. Water in which the plants had been grown did not promote degradation.

KEYWORDS: TNT, degradation, plant species, roots

INTRODUCTION

Residues of 2,4,6-trinitrotoluene (TNT) are found at sites of past production of explosives. The residues are harmful to many living organisms and have been known to even cause deaths in humans. There are presently many EPA-required clean-ups in progress. Although a lot of effort has gone into understanding the fate of TNT in the environment and into developing strategies for bioremediation, there is relatively little literature on the beneficial use of plants in bioremediation of TNT [1-12]. Certain aquatic plants are suggested to be able to reduce the TNT to compounds that are neither toxic nor mutagenic [9, 10].

In some locations, parts of the ecosystem have been slowly overwhelmed by the solid residues of trinitrotoluene, also known as TNT, that were left behind by the production of explosives. High concentrations of TNT are commonly found in soils within and surrounding sites of past explosive loading, handling, and packaging activities [2]. No higher plants grow in locations with high concentrations of TNT. The TNT, currently identified as a soil contaminant, is deadly to many organisms, both lower and higher animals [10].

Another problem with TNT arose from the use of large volumes of hot water to wash off the residual explosives. The water which became saturated with TNT was discharged into ditches. From there, the water often was then discharged into local streams [11]. Living in the streams and edges of oceans are several “indicator” aquatic organisms that include fresh water unicellular green algae, tidepool copepods, and oyster larvae, whose death rates drastically increase with concentrations of TNT greater than 5.0 mg/liter [12]. TNT is also toxic to fish, fungi, Gram-positive bacteria, rats, mice, and plants [3]. The problem has reached the point where all TNT manufacturing at U.S. arsenals was discontinued because they could not meet the EPA standards [6]. The focus now is on cleaning up the TNT-contaminated sites.

Lengthy exposure to TNT is severely detrimental to human health. Several health problems include liver injury and “marked changes in the hematopoietic system producing anemia” [11]. Also, TNT has been known to affect the survival of erythrocytes, liver function, and the lens of the eyes. In addition, there are records of deaths from toxic hepatitis and aplastic anemia caused by TNT exposure [4].

During the past few decades, studies have been done in an effort to solve the problems arising from TNT residues. During the 1970s, William D. Won and his associates used *Pseudomonas*-like organisms to degrade the TNT. According to Won, products from the breakdown of TNT appeared non-toxic and non-mutagenic [11]. In 1986, Palazzi and Legget [7] conducted an experiment with a terrestrial plant, nutsedge, grown hydroponically. Their purpose was to find out the effect of the plants on the TNT. They concluded that the plant took in the TNT and that the metabolites were formed within the plants. However TNT was rather toxic in this plant (*Cyperus exculentus* L.).

Currently, studies are mainly being done on the effects of enzymes and plants on TNT. In a recent study using enzymes from aquatic sediments, the conclusion was that the enzymes were capable of reducing the TNT to products that were less toxic and mutagenic. Both van Beelen [10] and Harvey [3] have concluded that 2-amino-4,6-dinitrotoluene or 4-amino-2,6-dinitrotoluene was the peak that appeared right before the TNT during HPLC separation. However, they were not able to determine which one it was since both 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotoluene appeared at the same time. They were able to identify the peak that appeared two peaks before the TNT (prior to the aminodinitrotoluene) as 2-nitro-4,6-diaminotoluene. It is believe that the enzymes found in sediments could have come from aquatic plants [10]. McCutcheon and his team believed that they have identified a few nonspecific enzymes that came from plants which can breakdown TNT. Nitroreductase is one of the enzymes found in 20% of the plants that were tested [9].

Phytoremediation is the term for using plants to remediate contaminants. Jimson weeds (*Datura innoxia* and *Datura quercofolia*) and a wild tomato species (*Lycopersicon peruvianum*) are types of plants that could be grown in soils contaminated with TNT up to a certain level [9]. Apparently, the plants absorb the TNT through their roots and detoxify them by reduction and acetylation. Poplar trees have been proven to be able to reduce herbicides in streams [5]. Alfalfa, corn, and soybean are some of the plants that have been used to clean up several contaminated sites composed of contaminants other than TNT [8]. It is suggested that these or similar crop plants may be useful for TNT cleanup [5, 9].

The purpose of the studies described here is to determine whether poplar trees, alfalfa, corn, and other species can decompose TNT. Sixteen genera of plants were tested to see whether they could degrade TNT. One set of experiments involved placing the roots of the plants into TNT solutions. Every few hours, samples were taken from solutions and tested for TNT disappearance by HPLC. Other sets of experiments involved placing cut-off roots, frozen roots, and ground-up roots into TNT solution to see whether they could reduce TNT.

According to engineers who are working on a program under the Defense Department, conventional technology could cost $1

trillion over the next thirty years [5]. For the moment, incineration is the technology that is used to remediate TNT-contaminated soil. However, incineration is very costly [1]. If plants can be grown at the TNT-contaminated sites, it would save our society billions of dollars in cleaning up the hazardous waste [5].

MATERIALS AND METHODS

HPLC analysis

A Beckman High Performance Liquid Chromatography (HPLC) with a Hamilton PRP-1 column was the main apparatus used to measure the disappearance of TNT. The UV detector was from Hitachi and was used at 238 nm, the best wavelength to detect TNT. Some degradation intermediates have lower absorbance at this wavelength but in the absence of a diode array detector, this was chosen as a compromise. Acetonitrile:water at a ratio of 65:35, with l mM KOH, was the chromatography solvent used to carry the sample from the injection point through the column to the UV detector. TNT and other compounds were detected at the wavelength 238 nm, and rates of TNT degradation are indicated as the decrease in peak height. Some studies were done using scanning spectrophotometers to confirm that the expected intermediates having increased absorbance at longer wavelength were in fact being formed.

TNT stock treatment solution

The TNT solution used for these studies consisted of 25 mg 2,4,6-trinitrotoluene, 500 ml distilled H_2O, and 25 ml of 20 mM potassium phosphate buffer, pH 6.85.

Chemicals

The 2,4,6-trinitrotoluene was purchased from CHEM Service. Standards of 2-amino-4,6 dinitrotoluene and 4-amino-2,6-dinitrotoluene were from Supelco. Chemicals for the Hoagland solution buffer for plant growth were reagent-grade from commercial sources. Acetonitrile was HPLC-grade obtained from Fisher Scientific. For HPLC, distilled, deionized water was used.

Plants

The following species were tested: *Sorghum bicolor* (sorghum), *Zea mays* (maize or corn), *Cyperus papyrus* (papyrus), *Typha latifolia* (cattails), *Glycine max* (soybean), *Medicago sativa* (alfalfa), *Helianthus annuus* (sunflower), *Tamarix parviflora* (salt cedar), *Populus deltoides x nigra* (hybrid poplar), *Brassica rapa* (Wisconsin Fastplants), *Lysopersicon esculentum* (tomato), *Arabidopsis*, *Alternanthera tricolor* (amaranthus or Joseph's Coat), *Nicotiana tabacum* (tobacco), *Cucurbita maxima* (pumpkin), and *Dioscoria batata* (sweet potato).

The plants were obtained from stores or collected locally and grown under ordinary fluorescent light or in a south-facing window. The room temperature was usually 24+/- 2ºC. Plants were grown with half-strength Hoagland solution, either hydroponically or in a support medium of perlite/vermiculite 1:1. Usually they were transferred to distilled water for a day prior to exposure to TNT so that free nitrate was depleted. Nitrate absorbs strongly at the wavelength of HPLC detection and interfered with observing triaminotoluene, the most reduced product of TNT degradation.

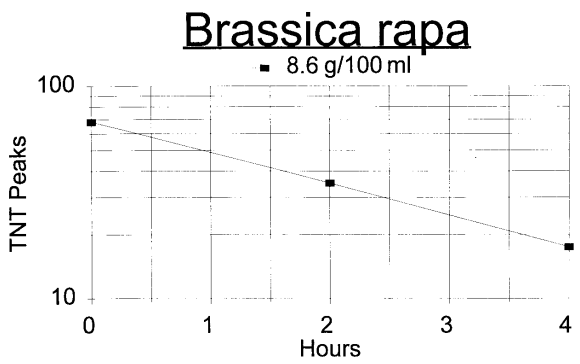

FIGURE 1. DECREASE IN TNT AS A FUNCTION OF TIME FOR *BRASSICA RAPA*.

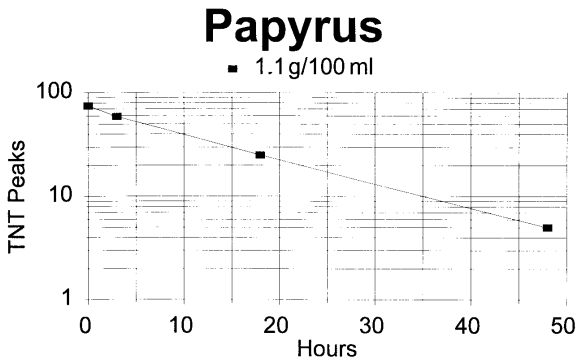

FIGURE 2. DECREASE IN TNT AS A FUNCTION OF TIME FOR PAPYRUS.

General treatment procedure using whole plants

Plant roots were rinsed under distilled water to remove excess support medium. The plant was placed in a beaker and the beaker was filled with enough water to cover up the top of the roots, after which it was allowed to sit for a few days so the plant could adjust to living in water. The beaker was refilled with TNT solution, usually at 50 mg/l. The beaker was wrapped with a black plastic wrapper to keep out the light. Every few hours, a sample of the solution was taken with a filtered pipette, or 0.22 μm sterile filter. A portion (25 μl) of the sample was run through the HPLC. When all traces of TNT were gone or nearly gone, the roots were cut and their weight was measured after they were blotted dry. The remainder of the plant was placed back in the Hoagland solution to grow back its roots.

General procedure using roots only

Approximately 1 g of roots were cut from a rinsed plant and weighed exactly after blotting dry. The roots were placed into a beaker filled with 50 ml of TNT solution. The beaker was wrapped, then samples were taken and analyzed as above.

RESULTS

Before the start of each set of experiments, a sample of TNT was tested through the HPLC to find out the original height of the TNT peak. Plants were placed into TNT solutions and samples were collected every few hours. The TNT peaks were compared to the original peak in order to calculate the rate of TNT disappearance. As the amount of TNT present in the solution decreased, other, more rapidly eluting, peaks appeared. These were tentatively identified as the aminodinitro and diaminonitrotoluenes and triaminotoluene on the basis of mobility in HPLC. At later times a peak appeared co-eluting with triaminotoluene.

Figures 1-3 show typical results for the decrease in TNT as a function of time. The response appears to be nearer to first order than linear, indicating that the apparent Km of the enzyme is very high or diffusion of TNT to the site of reaction is limiting. This same behavior was observed with aquatic plants by the EPA group at Athens, GA [8]. To reduce the data to a comparable basis we used similar amounts of plant tissue per volume of TNT and estimated the half-time from semi-log plots (log TNT vs. time). Most experiments were done with an initial TNT concentration of about 50 mg/l but rates are

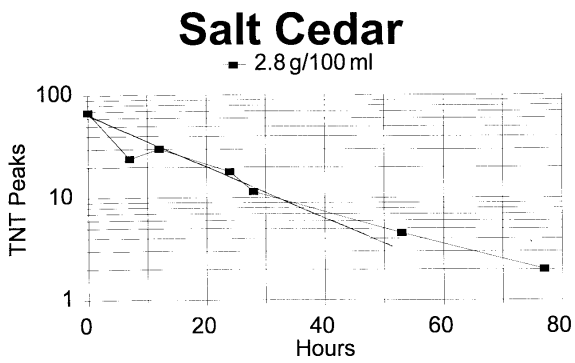

FIGURE 3. DECREASE IN TNT AS A FUNCTION OF TIME FOR SALT CEDAR.

indicated as a percent change of concentration based on HPLC peak height. Samples were taken at intervals up to several days and all rates are based on estimates of multiple times of sampling.

In Table 1, the amount of time taken by a specified amount of plant per unit volume of TNT to degrade 50% of the TNT is compared. Some plants were faster than others at reducing TNT. From Table 1, *Zea mays, Cyperus papyrus, Medicago sativa, Alternanthera tricolor, Dioscoria batata,* and *Cucurbita maxima* appeared to be the plants which could degrade TNT the quickest. *Helianthus annuus* and *Typha latifolia* seemed to be slowest. For some of the plants, the time it took to degrade 50% of the TNT differed greatly in different experiments. For example, with sorghum the half-time was 12 hr/g/100 ml in one case and 30 hr/g/100 ml to degrade TNT. With soybeans the halftimes were 9 and 50 hr/g/100 ml. Plants were tested at different ages, some with initial roots, others with regrown roots in hydroponic culture. We do not yet know the relationship of root age or nutritional status to the expression of the nitroreductase activity. There appeared to be no simple, obvious relationship.

Three methods were used to determine whether the roots were where the TNT was absorbed into and converted into other compounds. First, roots cut from plants were tested in TNT solutions. Second, the plant roots were frozen to see whether the reaction can occur in roots whose activities were stopped for about a day and whose cells had been freeze-damaged. Third, TNT solution was added to ground-up roots. In all three methods, the roots were able to degrade TNT which suggests that the enzymes were in or associated with the

TABLE 1. AMOUNT OF TIME IT TOOK TO DEGRADE 50% OF THE TNT.

Plant Species & Sample Type	hr/g/100 ml
Typha latifolia #1	52
Plant #2	58
Sorghum bicolor #1	12
Plant #2	30
Zea mays #1	9
Plant #2	17
Glycine max #1	50
Plant #2	9
Tamarix parviflora	28
Cyperus papyrus	12
Medicago sativa	35
Brassica rapa	17
Alternanthera tricolor	17
Populus deltoides x nigra #1	20
Plant #2	8
Roots alone #1	10
Roots alone #2	10
Roots alone #3	50
Frozen roots	50
Lysopersicon esculentum	2.5
Frozen roots	36
Ground-up roots	40
Filtered ground-up roots	40
Dioscoria batata	16
Frozen roots	15
Helianthus annuus	32
Filtered ground-up roots	75
Cucurbita maxima	15
Roots alone	5
Nicotiana tabacum	45
Arabidopsis	>100

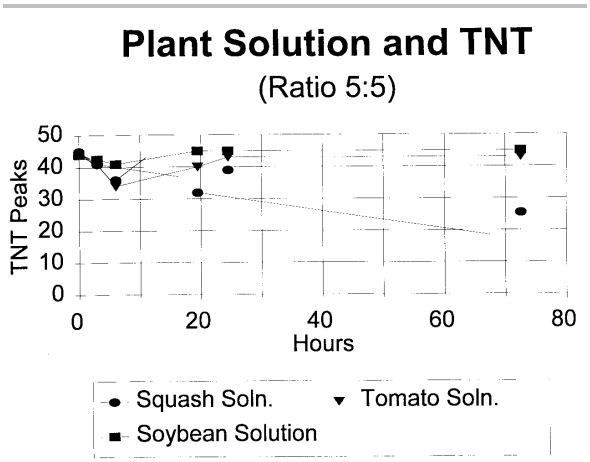

FIGURE 4. ADDITION OF TNT TO PLANT SOLUTIONS SHOWED THAT THE ENZYMES WERE MOSTLY IN THE ROOTS.

roots. However, this did not establish whether the reaction occurred in the roots or in the solutions surrounding the roots. To determine whether the enzymes were inside the roots or if the roots were leaking the enzymes out, TNT solutions were added to the solutions in which the plants had been grown and removed. At first, they showed some signs of activities but then all activities stopped which suggests that the enzymes, for the most part, were in the roots (Figure 4).

We observed several HPLC peaks that appeared while the TNT was disappearing. The peaks have not been completely identified. The peak which appears right before the TNT peak could be 2-amino-4,6-dinitrotoluene or 4-amino-2,6-dinitrotoluene. When samples of the two chemicals were separately analyzed, each caused a peak to appear at the same place as the experimental peak that appears right before the TNT peak [10]. It was not possible to distinguish conclusively which one it was and whether one or both could be that peak. Mass spectrometry will be needed to resolve this question. The end product which is triaminotoluene may be the first peak eluted during chromatography. When a sample of the triaminotoluene was tested, it appeared at the same time as the first peak. However, nitrate peaks also appear at the same place. So would other polar UV-absorbing materials. The peak that appears after TAT and before the aminodinitrotoluene peak could possibly be 2-nitro-4,6-diaminotoluene, based on the work of van Beelen and Burris [10].

To insure that the experimental peaks were actually degradation products, two types of controls for the experiments were conducted. The first type of control was used to test whether the plants were leaking out UV-absorbing compounds. This test was accomplished by placing the plants into distilled water before or after TNT treatment. Samples of the water were taken and run through the HPLC. Generally, no peaks were observed, which means that the plants were not leaking out any measurable amounts of UV-absorbing compounds into the solution.

The second control was used to test for the natural decomposition of TNT. TNT was placed into a dark environment and observed over an extended period of time. No major changes occurred over time. When exposed to light, the solution turned pink and TNT disappeared. However, this process was not accompanied by accumulation of peaks attributable to intermediates observed in the presence of plants.

DISCUSSION

All of the tested species showed degradation activities which suggests that the activities were occurring in the roots. Isolated roots generally had less activity than intact plants. Ground-up roots showed even less activity initially but there was an increase with time. To try to rule out microbial contamination of such extracts, samples were centrifuged at

high speed and then filtered through 0.22 μm filters. Activity in these samples, which were expected to be sterile, increased with time. One last set of experiments involved adding TNT solutions to the solutions in which the plants had been grown. There was very little activity in this liquid, which suggests that the enzymes or required energy sources, for the most part, were in the roots. This means that it might be possible to purify the enzymes that are in the roots.

All the plants that were tested, with the possible exception of *Arabidopsis*, were able to degrade TNT into other compounds. The experiments indicated that the roots took in the TNT and reduced it to other compounds. Inside the roots could be the enzymes that other researchers reported were in plants and were responsible for converting TNT into both less toxic and non-toxic products. The products could be 2-amino-4,6-dinitrotoluene or 4-amino-2,6-dinitrotoluene, 2-nitro-4,6-diaminotoluene, and triaminotoluene. However, the products have not been fully identified at this time.

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